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High Energy, Ultrashort Pulse Green Laser-Light Exposure of Cultured Human Cells Yields Evidence of DNA Damage

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The use of laser light for targeting devices and weapons has sharply increased the likelihood that aircrew and support personnel will be exposed to laser light during operations. The increased potential for exposure of humans highlights the need for scientifically-based safety standards for laser exposure at the ultrashort pulse lengths. Current safety standards are largely extrapolations of exposure limits at longer pulse lengths using a minimal visible lesion endpoint in the Rhesus monkey retinal model. A non-animal model for assessing laser-light damage to tissue, particularly human, is necessary for obvious scientific, political, and fiduciary reasons. We assessed the sublethal insult to human cells using a tissue culture system for specific genes that have been shown to be important in several biological processes that could lead to cancer or cell death. Using the CAT-Tox (L) (Xenometrix, Inc.) assay, it appears that green (532 nm), picosecond pulses of laser light is sensed and induces several stress response genes, including FOS, a proto-oncogene, in a roughly dose dependent fashion. Numerous other genes were also induced harbingering the presence of DNA damage. This approach provides insight into a more global methodology for characterizing environmental stressors via genetic profiling.

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The use of laser light for targeting devices and weapons has sharply increased the likelihood that aircrew and support personnel will be exposed to laser light during operations. The increased potential for exposure of humans highlights the need for scientifically-based safety standards for laser exposure at the ultrashort pulse lengths. Current safety standards are largely extrapolations of exposure limits at longer pulse lengths using a minimal visible lesion endpoint in the Rhesus monkey retinal model. A non-animal model for assessing laser-light damage to tissue, particularly human, is necessary for obvious scientific, political, and fiduciary reasons. We assessed the sublethal insult to human cells using a tissue culture system for specific genes that have been shown to be important in several biological processes that could lead to cancer or cell death. Using the CAT-Tox (L) (Xenometrix, Inc.) assay, it appears that green (532 nm), picosecond pulses of laser light is sensed and induces several stress response genes, including FOS, a proto-oncogene, in a roughly dose dependent fashion. Numerous other genes were also induced harbingering the presence of DNA damage. This approach provides insight into a more global methodology for characterizing environmental stressors via genetic profiling.
Key Words: laser bioeffects, gene profile, laser safety

Background

Military and civilian technology of the 21st century will increasingly rely on the use of laser light, and thus increase the chances that personnel will be intentionally or accidentally optically exposed. Current safety standards for laser light exposure to the eye are based largely on whole animal minimal visible retinal lesion studies, and do not take into account the possibility of subtle sub-lethal long term effects which may become manifest long after acute treatment. Furthermore, current treatment of laser-exposed patients is concluded after visible lesions abate. And there is not a thorough scientific understanding of the laser-light damage mechanisms at the cell and molecular level. Therefore, it would be of great benefit to develop, validate, and genetically engineer a tissue culture based methodology with cell lines possessing qualitatively and quantitatively sensitive damage-induced reporter gene systems.

Such cell lines would provide an *in vitro* model of the laser-tissue interaction in the eye, which could serve as the test bed for a variety of experiments leading to the development of sub-lethal laser exposure safety limits. The outermost layer of the retina, the Retinal Pigment Epithelium (RPE), plays a critical role in the physiology of the underlying photoreceptors(1). In order to engineer a retinal pigment epithelial cell line, we must first investigate the generic genetic response, if any, of human cells to laser-light exposure. In this experiment, using a battery of 13

known damage-induced reporter genes, we assessed the genetic response of human cells grown in a tissue culture format to laser-light insult. The development of a non-animal model for assessing laser-light damage to living tissue, particularly human, is necessary for obvious scientific, socio-political and fiduciary reasons and we believe that it has become technologically possible.

Materials and methods

General description:

The purpose of this study was to investigate the gene activity induced in human cells by high energy, ultrashort pulse laser-light exposure. The CAT-Tox (L) assay, developed by Xenometrix, Inc. (Boulder, CO), is designed to detect transcriptional responses to a variety of compounds including DNA damaging agents and oxidative stressors in human liver cells. This gene profile assay uses a human liver cell line (HepG2) and 13 mammalian gene reporter constructs driving expression of the chloramphenicol acetyltransferase (CAT) reporter gene. This assay was selected because it was technically appropriate, commercially available and relatively easy to adapt to laser bioeffects investigation.

In this particular series of experiments, a battery of 13 human stress response genes [briefly described in USAFA-TR-1999-01(2)] in reporter gene constructs known to be induced by various types of cellular stress or damage were used to assess the bioeffects of laser light exposure on human cells at the cell and molecular level. This mammalian gene profile assay is capable of measuring differential gene expression in the human hepatoma cell line, HepG2. The thirteen different recombinant human liver cell lines were generated by creating stable transfectants of different mammalian promoter-CAT gene fusions. The activity of a given promoter is quantified simply by the accumulation of CAT protein, measured using a standard CAT ELISA (Enzyme Linked Immuno-Sorbent Assay) detection system.

A broad range of promoters responsive to DNA damage, heavy metal ions, protein denaturants, aromatic hydrocarbons, retinoids, and changes in intracellular cyclic AMP levels have been included in the assay. In some cases specific response elements are monitored, permitting fine analysis of stress-regulated gene expression.

The gene profile assay [CAT-Tox (L)] has been designed to use a 96-well microtiter plate format. This system gives simultaneous dose-response information at five different exposures for the 13 recombinant cell lines. The assay yields results in 24 to 48 hours. The results are displayed in histogram form as a XenoMatrix^{TM}. The assay also includes the parental HepG2 line for the measurement of cytotoxicity. The assay can distinguish subtle differences among closely related effects, and can indicate molecular mechanisms of sub-lethal cellular injury.

Exposure:

For the CAT-Tox (L) assays the cells were divided into the following groups. Two rows of cells per 96-well plate were controls (non-lased cells-Treatment A). The adjacent two rows of cells (Treatment B) were not lased directly, but were assayed to examine the effect, if any, of

reflected/refracted laser light received while the adjacent wells were lased. This had particular significance in valuing the control cells. The remainder of the rows, two by two, were exposed to various energies of laser-light using the 532 nm wavelength of the Nd-YAG laser (Quantel International, YG501) pulsing at 10 Hz at a pulse width of 37 picoseconds (ps). The beam was shaped to as uniformly as possible fill the bottom of the well. The lowest exposure was one second delivering 22 mJ per pulse at 10 Hz delivering a total of 220 mJ at the surface of the tissue culture media in the well. The highest dosage was 60 seconds (s) at 22 mJ per pulse at 10 Hz yielding 13200 mJ on target. All of the exposures were assayed at 24 hours post- exposure. All pulses were delivered to the surface of 50 microliters of growth media in a 6 mm well in 96-well polystyrene (Falcon, 3072) plates containing a confluent monolayer of cells on the bottom of the well. The chart immediately below indicates the exposure and assay regimen for the data displayed in Figure 1.

Treatment	Exposure Duration (s)	mJ per Pulse	Total Incident Energy (mJ)	Post Exposure Assay Time (hr)
A (control)	0	0	0	24
B	0	0	0	24
C	1	22	220	24
D	10	22	2200	24
E	30	22	6600	24
F	60	22	13200	24

Gene profile assay:

We adapted the manufacturer's protocol to assess the effects of laser-light exposure and they were done in triplicate. The assay involved 13 stably transfected human liver cell lines, each containing a unique stress-responsive promoter or response element fused to the CAT reporter gene. In each genetically induced cell line, the CAT reporter gene was transcribed and subsequently translated. CAT production is detected by an ELISA methodology yielding a quantitative measure of the stress gene induction expressed as fold induction compared to the control. Those cell lines without CAT protein production will not show gene induction above the control (non-lased cells). One Gene Profile Assay was performed, using a human liver cell line (HepG2), and 13 mammalian gene reporter constructs driving expression of the CAT gene. The assay was performed with concurrent negative controls, and assayed separately with known positive control exposures. The assays performed within acceptable limits for both positive and negative controls.

Assay procedure: A shortened version of the assay protocol is as follows.

1. The 13 recombinant cell lines and the parental HepG2 cell line are plated, one row each, over two 96-well microtiter plates and grown to confluence.
2. The cell lines are dosed at five exposures and incubated at 37°C, 5% CO_2 for 24 or 48 hrs.
3. After the post-exposure incubation period the cells are washed two times and lysed with a detergent based buffer to release total cellular protein.
4. An aliquot of the total protein is transferred to 96-well microtiter plates containing Bradford protein dye. Incubation of the protein with the protein dye creates a color

change that can be measured at optical density $(OD)_{600}$. This reading serves as a normalization factor for total cellular protein from well to well in the assay.

5. The remaining cellular protein is transferred to 96-well plates containing polyclonal anti-CAT antibodies. A standard sandwich ELISA is performed and in the final step horseradish peroxidase catalyzes a color change reaction that can be measured at OD_{405}.
6. The parental HepG2 cell line that was dosed in the same manner as the 13 recombinant lines is used to perform an 3-(4-5-dimethlythiazol-2yl)-2,5 diphenlytetrazolium bromide (MTT) reduction-based cellular viability assay. The results of this assay can be monitored at OD_{550}.
7. Xenometrix software uses the OD_{600} and OD_{405} readings to calculate the transcriptional fold induction for each recombinant cell line at each test exposure. The software also converts the OD_{550} to cellular viability percentages.

All plates containing the control and experimental cells were measured in an automated microplate reader (Bio-Kinetics, EL312e) which read their optical density (endpoint of ELISA) as a measure of CAT production or gene expression. This data was then electronically transferred to a computer database and eventually tabulated in graphic form. Normally three trials were conducted and the results were averaged (graphically represented in Figure 1). The assay allowed us to gather qualitative (which genes were activated) and quantitative (fold induction) data.

Data analysis

Cell viability:

Cell viability is calculated as the percent of the cells surviving at the assay time in the dosed samples versus the no dose control. In the gene profile assay, viabilities are recorded based on the results of MTT viability assays performed concurrent with the assay.

Gene expression calculations:

Gene expression is measured as the fold induction. That is the fold (multiple) increase of a construct at concentration n is determined by dividing the construct activity at each dose, n, by the activity at the zero dose. The levels of gene induction are expressed as multiples of basal or background values (zero dose). In the gene profile assay, background activity values are normalized and represented as 1.0 fold induction.

Statistical analysis

Unequal variance comparison t-tests and multiple comparison with a control case (The Dunnett test) were used to determine significance (at the $\alpha = 0.05$ level) of activity at each dose (the corresponding fold increase must be greater than 1.0). The unequal variance t-tests was performed to test the null hypothesis for each concentration of a compound in comparison to the zero-dose control. The following formula was used for the t-tests:

Hypothesis: H_0:mean activity at dose i=mean activity at zero dose
H_1:mean activity at dose i>mean activity at zero dose

When $t > t_{a,v}$ at the $\alpha = 0.05$ significance level, H_0 was rejected and there exists a significant difference between the man activity at dose *i* and the activity at the zero dose.

The unequal variance *t*-tests provide the analyses corresponding to the different dose levels *vs.* the zero dose control comparisons. However, assuming each of the separate comparisons is performed at significant level α, each *t*-test is subject to a possible false positive ("type I") error rate equal to $100\alpha\%$. When multiple comparisons are conducted on the same set of data, this error rate applies to *each* comparison, and may be considerably inflated using simple *t*-test comparisons. To correct for this multiplicity, the test statistics must be adjusted to bring their experiment-wise error rates back to α. This was accomplished by incorporating simultaneous inferences on multiple comparisons. Thus we used the Dunnett test (or Multiple Comparison with a Control Case).

The statistical analysis was performed through both the unequal variance t-test and Dunnett's test. The conclusive statistical significance was based on either of these tests demonstrating significance and the calculated fold induction was above 1.0.

Results

Cell cytotoxicity:

The cell viability portion of the assay shown along the left wall on the graph on Figure 1 indicates that the parent cell line withstood pulses of 532 nm laser light with at or greater than 100% viability for all exposures. On Figure 1 each unit shown vertically along the left wall represents 10% viability when reading viability (i.e. 5 equals 50% viability). Viability increased markedly in exposure treatments E and F as shown graphically on the left wall of Figure 1 with Treatment F spurring the cells to 140% viability. It is important to note that the MTT viability test used is based on cell respiration (ATP production) rates and may actually reveal an underlying cellular stress response which is obviously counter-intuitive to the normal interpretation of this assay.

Human stress gene promoter induction:

The controls in this experiment were the identical cell lines that were not exposed to the laser light beam. Appropriately, these lines did not show fold induction (vertically along the left wall) in the assay as seen in Figure 1, and are used as a baseline for the treated cells. One of the stress reporter-gene cell lines, FOS(2,3,4), in this experiment showed a roughly dose-dependent induction, while six others, (GADD 45(5), GRP78(6), HMTIIA(7,8), CYPIA1(9,10), GADD153(11), NFkBRE(12,13) and HSP70(14,15)) showed irregular and weak responses. The remaining constructs did not exhibit any marked stress response. However, only GRP78, GADD45 and NFkBRE reached statistical significance as shown in Figure 2. It was also noted that only the highest energy exposure level in the 24-hr post-treatment assays were capable of significant stress gene induction.

Data interpretation

The results strongly indicate several important findings. First that human cells, liver in this case, have the ability to "sense" ultrashort pulse high-energy laser light. Secondly, human cells can respond to sublethal laser light insult using, at a minimum, some of the same stress response genes previously characterized in response to other cellular stressors. The nearly dose-dependent response exhibited in the FOS constructs was a particularly significant result. The FOS construct activation indicates possible DNA damage, which could have profound implications to the safety standards for laser exposure, since this implies subtle long-term effects such as carcinogenesis as FOS is a proto-oncogene. The NFkBRE, induced to a significant level in the highest exposure and to lower levels in several other exposures, has been shown to be involved in the inflammatory response and the apoptosis regulatory pathway. Apoptosis is defined as programmed cell death or cell suicide. This finding is concordant with and possibly explains the "biological magnification" of retinal lesions non-existent at 1 hr post-exposure but present at 24 hours post-exposure. DNA damage is also a requisite for the induction of the GADD genes which are almost universally involved in repairing DNA damage. As noted above GADD45 induction reached statistical significance while GADD153 did not due to greater inter-sample variation although around a higher mean. These results are also consistent with the findings of Leavitt, et.al (16) who demonstrated a 10-fold increase in mutagenesis in Chinese Hamster Ovary cells after 532 nm laser light exposure. These low level construct inductions could signal DNA mutagenesis or other forms of aberrant gene activation. The GRP78 gene induction indicates possible heat stress and potential intracellular protein denaturation. Since the HSP70 gene was induced only in the 48-hour assays, it appears the heat stress was slight and easily remediated by cellular repair mechanisms thus probably not a major intracellular perturbation. The induction of HMTIIA is a bit of an enigma as it is normally affected by the presence of heavy metals, but it can also be induced by glucocorticoids in mammals (8) or possibly an unknown regulatory pathway. CYPIA1 expression is also a bit confounding since it normally responds to exogenous chemicals and drugs (10) as part of the oxidative metabolic degradation process. It may indicate the existence of post-exposure photochemical by-products that mimic an Ah (aryl hydrocarbon) ligand. In summary, it appears that there are multiple genetic indicators of DNA damage in the cells of this tissue culture based assay, as well as other cellular stress responses. Although DNA damage hardly seems possible as result of green light exposure. It, however, has been shown that some biological molecules, DNA for example (17), apparently have the ability to absorb multiple photons/quanta of light energy and allow frequency up-conversion of the light to the next higher energy harmonic. If indeed this is happening within the exposed cells then the 532 nm light would be converted to 266 nm light which is in the wavelength range of untraviolet (UV) light. The DNA damaging capability of UV light has been extensively demonstrated (18) and is the most likely DNA damaging agent in these experiments.

Conclusion

These results will point us in the proper direction for development of an immortalized RPE cell line genetically engineered with appropriate CAT-producing stress reporter-gene constructs. At this juncture we can say that any *in vitro* laser tissue damage assessment system should include

DNA damage constructs. Cell damage repair genes such as those coded for by the GRP78, NFkBRE and the proto-oncogene, FOS, should also be considered for incorporation. Such constructs will provide a qualitative basis as well as a quantitative measure for damage in the retinal cells, so that more sophisticated understanding of laser induced damage at the cell and molecular level can be elucidated.

From a more global perspective, these findings provide a "proof of concept" that gene profiling technologies can be extremely efficacious in approaching the problem of laser-tissue interaction from the biological perspective. Gene profiling can provide insight into the type and degree of laser induced damage at the cell and molecular level as well as the damage thresholds for induction of repair metabolism or promotion of apoptosis or necrosis. The medical ramifications of these technologies in the context of prophylaxis and treatment are obvious.

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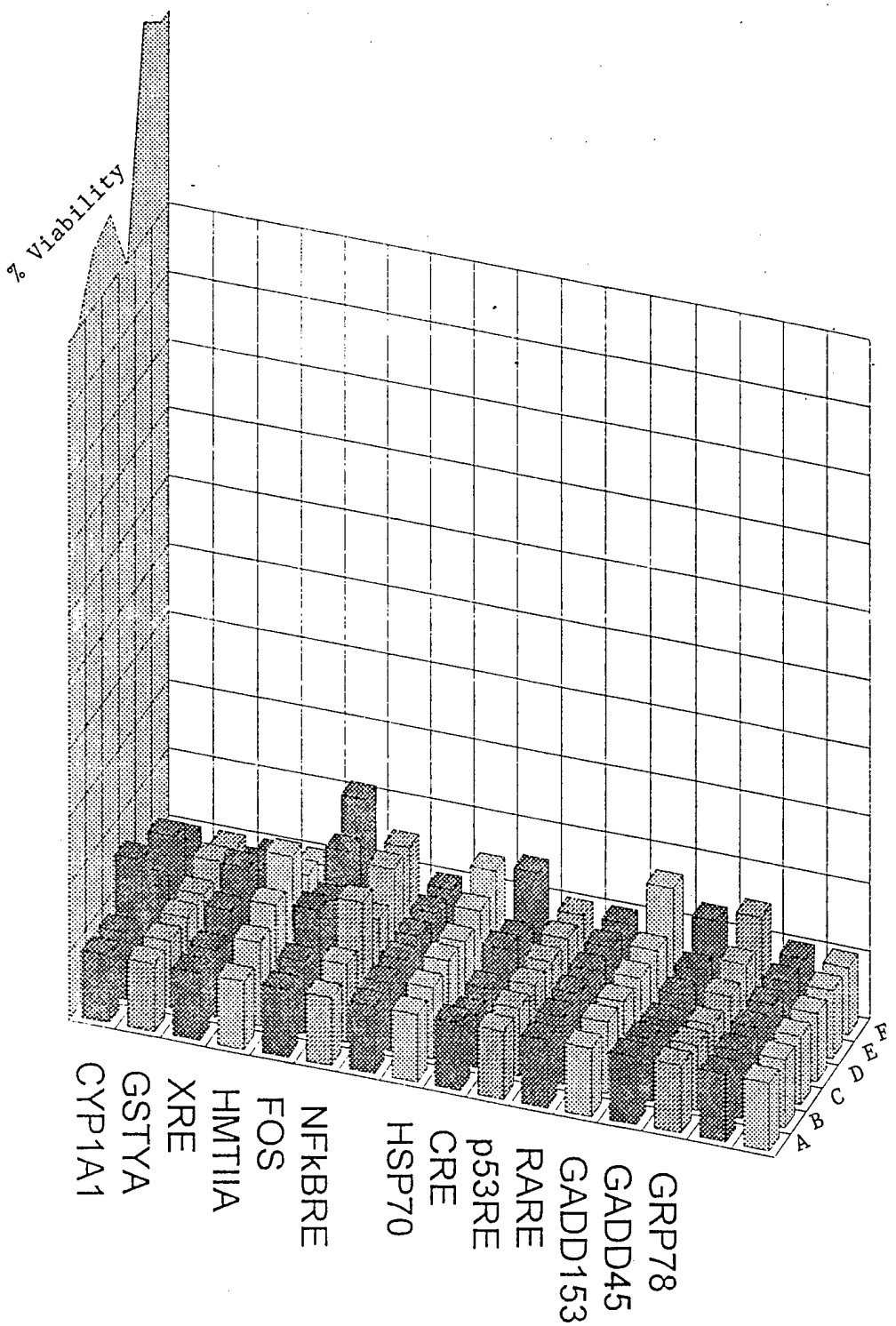

FIG. 1. Fold Induction of 13 Human Stress Response genes after laser-light exposure. The vertical scale represents fold induction of the genes designated in the foreground and percent survival the parental cell line as shown on the left wall. The exposure treatments are along the right floor (A-F) and described in Table 1.

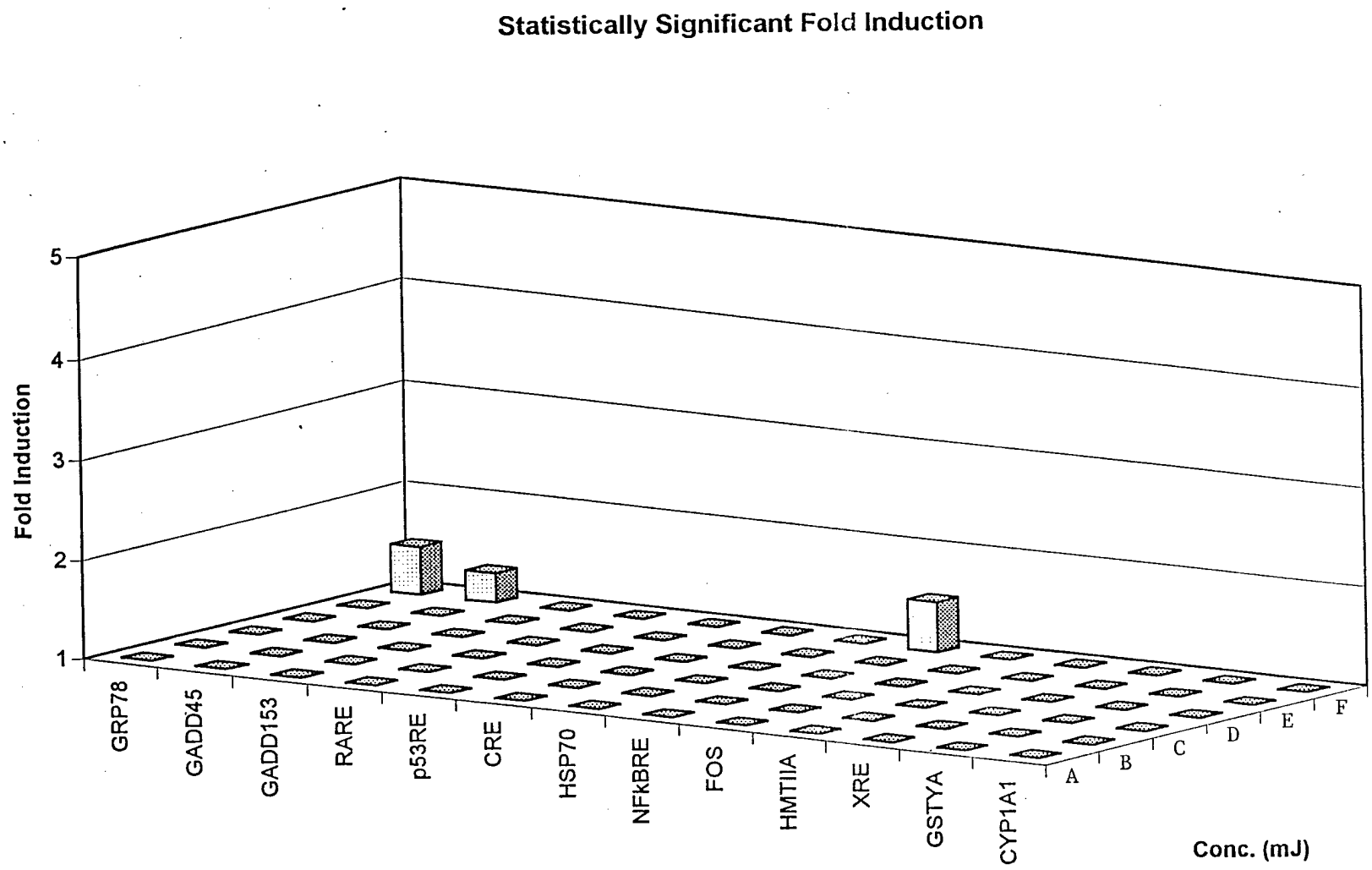

FIG. 2: Statistically Significant Fold Induction of the data presented in Figure 1.